SUPPLEMENTARY DATA

Functional Impact of Heterogeneous Nuclear Ribonucleoprotein A2/B1 in Smooth Muscle Differentiation from Stem Cells and Chick embryonic arteriogenesis

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Detailed Methods & Materials

Materials. Antibodies against hnRNP A2/B1 (mouse, EF-67, sc-53531), SRF (rabbit, G-20, sc-335) and MEF2c (goat, C-17, sc-13268) were purchased from Santa Cruz Biotech, USA. Antibody against Smooth Muscle Myosin Heavy Chain (SM-MHC) was from AbD Serotec (Rabbit, AHP1117). Antibodies against HA (H6908), α-tubulin (mouse), histone 4 (Rabbit), hnRNPA2/B1 (mouse, clone DP3B3) and monoclonal anti-α smooth muscle actin (SMαA) (Clone 1A4, A5228) were from Sigma. All secondary antibodies were from Dako, Denmark. Other materials used in this study were purchased from Sigma unless specifically indicated.

Cell culture and SMC differentiation. Detailed protocols for mouse embryonic stem cells (mESCs) (ES-D3 cell line, CRL-1934; ATCC, Manassas, USA) culture and SMC differentiation were described in our previous studies(1-6). Undifferentiated ES cells were seeded on mouse collagen IV (5μg/ml)-coated flasks or plates in differentiation medium [DM, MEM alpha medium (Gibco) supplemented with 10% FBS, 0.05mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 3 to 7 days prior to further treatment. The medium was refreshed every other day.

Immunoblotting. Cells were harvested and lysed in lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0) supplemented with protease inhibitors and 0.5% Triton by sonication for whole cell lysate. Cell nuclear or cytoplasmic fractions were lysed separately in Hypotonic buffer (10 mM HEPES-KOH pH 7.2, 1.5 mM MgCl2, 10 mM KCl) or High-salt buffer (20 mM HEPES-KOH pH 7.2, 25% Glycerol, 1.5 mM MgCl2, 420 mM KCl, 0.2 mM EDTA) supplemented with protease inhibitors and 0.5% NP-40. 40 μg of protein was separated by SDS-PAGE with 4%~20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and subjected to standard Western blot analysis. In some experiments, the blots were subjected to densitometric analysis with Image J software. Relative protein expression level was defined as the ratio of target protein expression level to α-tubulin expression level with that of the control sample set as 1.0.

Indirect immunofluorescent staining for cells. Indirect immunofluorescent assay was performed as previously described (1,2). Cells were labelled with isotype IgG

control or antibodies against hnRNPA2/B1 (1:100; EF-67), and visualized using appropriate secondary antibodies conjugated with tetramethylrhodamine isomer R (TRITC, DAKO). Cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) and mounted in Fluoromount-G (Cytomation; DAKO, Glostrup, Denmark). Images were examined using SP5 confocal microscope with Plan-NEOFLUAR 63x objective lenses and Leica TCS Sp5 software (Leica, Germany) at room temperature, and were processed with Photoshop software (Adobe).

Real time quantitative PCR. Real-time quantitative PCR (RT-qPCR) was performed as previously described (2). Total RNA was extracted from cells using RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using an Improm-II^{TM} RT kit (Promega, Madison, WI, USA) with RNase inhibitor (Promega), and Random primers (Promega). Simultaneous RT reactions were performed without the addition of reverse transcriptase to control the possible transcription of contaminating genomic DNA. Primers were designed using Primer Express software (Applied Biosystems). Relative mRNA expression level was defined as the ratio of target gene expression level to 18S expression level with that of the control sample set as 1.0.

hnRNPA2/B1 expression plasmids generation. Mouse and Gallus full length hnRNPA2/B1 gene was amplified by RT-PCR from day 3 differentiating ES cells and chick embryos with primer set as shown in **Table S1** and cloned into Kpn I/Pst I sites of the pCMV5-HA and pSPT18 expression vector, designated as pCMV5-HA-hnRNPA2/B1 and pSPT18-hnRNPA2/B1. All vectors were verified by DNA sequencing.

Nucleofection. Nucleofection was performed as previously described (2). For transient transfection, pCMV5-HA-hnRNPA2/B1 was introduced into undifferentiated ES cells using mouse ES cell nucleofection kit (amaxa, VPH-1001) with nucleofector II (amaxa, Germany) according to the manufacturer's instructions. Transfected cells were plated on dishes coated with 5μg/ml of collagen IV and cultured for 3-4 days in the DM to allow SMC differentiation.

siRNA experiments. Cbx3 siRNA (sc-35590), hnRNPA2/B1 siRNA-1 (Sc-43842), and control siRNA-C (sc-37007) were purchased from Santa Cruz Biotech. Control siRNA (SIC001-10NMOL) and hnRNPA2/B1 siRNA-2 (siRNA ID: SASI_Mm01_00200165) were purchased from Sigma. ES cells were cultured on collagen IV-coated 6-well plates for 2-4 days, and 10 μl of 10μM siRNA (final transfection concentration of siRNAs: 33nM) was introduced using siIMPORTER transfection reagents (Millipore) according to the protocol provided. Cells were harvested at 48 or 72 hours after transfection and real-time RT-PCR and Western blot analysis were performed.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assays were performed as previously described (4-6). Differentiating ES cells transfected with pCMV5 or pCMV5-hnRNPA2/B1 were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. The medium was removed, and cells were harvested and sonicated. The sheared samples were diluted into 1 ml immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.2, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and immunoprecipitation was conducted with antibody raised against hnRNP A2/B1, together with single-strand salmon sperm DNA saturated with protein-G-Sepharose beads. Normal IgG was used as a control. The immunoprecipitates were eluted from the beads using 100 μl elution buffer (50 mM NaHCO3, 1% SDS). A total of 200 μl proteinase K solution was added to a total elution volume of 300 μl and incubated at 60°C overnight. Immunoprecipitaed DNA was extracted, purified, and then used to amplify target DNA sequences by RT-qPCR. Relative DNA level was defined as the ratio of immunoprecipitated promoter DNA level to its input level with that of the control sample (pCMV5) set as 1.0. Target DNAs were almost undetectable in the normal IgG control samples. The data was obtained from three independent experiments.

Chick Embryo Cultivation and Staging. Fertilized chick eggs were obtained from Joyce and Hill (Farm) and incubated at 38.5°C in a humidified incubator. Hamburger and Hamilton (HH) staging was applied throughout the study(7,8).

Whole-mount immunohistochemistry. Chick embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight. After graded dehydration in Methanol, the embryos were incubated with freshly prepared Methanol:DMSO:H_2O_2 (4:1:1) at room temperature for 5 hours. Then the embryos were rehydrated in a series of 50% Methanol/PBS, PBS, and blocked with PBSMT (2% non-fat milk powder, 0.5% Triton X-100 in PBS). Subsequently, the embryos were incubated with mouse anti-SMαA (1:2000, Sigma), anti-Cbx3 (1:500, Santa Cruze) in PBSMT at 4ºC overnight. Mouse IgG was used at same concentration as control (**data not shown**). Followed by HRP conjugated secondary antibody and DAB staining, the embryos were fixed in 4% PFA for 10 mins, rinsed in 25%, 50% glycerol/PBS, and mounted with 75% glycerol/PBS for observation.

In situ **hybridization.** The full-length cDNAs for hnRNPA2/B1 were made by PCR using specific primers and subcloned into pSP18 vector. The dig-labeled anti-sense RNA probe for hnRNPA2/B1 was then synthesized using SP6 and T7 RNA polymerase (DIG RNA Labeling Kit, Roche). Chick embryos were fixed in 4% PFA in PBS overnight, and hybridized with hnRNPA2/B1 anti-sense RNA probes. Sense RNA probe was also synthesized and used as control (data not shown). Following hybridisation, the embryos were extensively rinsed and the DIG-labelled probes were detected using NBT/BCIP (Boehringer) as substrate for the anti-DIG-antibody-coupled alkaline phosphatase (protocol modified from(9,10)).

In vivo **electroporation on chick embryos.** *In vivo* electroporation carried out on chick embryos was performed as previously described but with some modifications (11-13). Eggs were incubated in a humidified incubator at 38°C for around 36 hours. A window was opened at the top of the egg shell using a blade, and embryos at HH 10/10$^-$/10+ were electroperated with hnRNPA2/B1 morpholino (1μM) or control morpholino (1μM) as control. Electroperator was set at 7.5 volts, 4 pulses, length of 50ms and interval of 1000ms. Oligonucleotides were mixed with 60% sucrose and fast green to aid visualization before electroporation. After electroporation, the eggs were sealed well and placed back into the incubator, window side up and incubated until the required developmental stages.

Immunofluorescent staining for sections. Chick embryos were fixed in 4% formaldehyde (FA) in PBS for 1 hour, rinsed in PBS and dehydrated using 5% sucrose, 20% sucrose (Sigma) in PBS at 4°C overnight. Then the embryos were embedded in 7.5% gelatin (Sigma G2500, 300-Bloom) in 15% Sucrose/PBS). Sections were cut at 10 um, picked up on Superfrost Microscope Slide (Thermo Scientific), air dried for staining or stored in -80 °C. Prior to antibody staining, gelatin was removed in 42°C PBS for 10 mins. The sections were then rinsed in PBS and blocked with 10% Goat Serum in PBS (Dako) for 1 hr at room temperature in a humid chamber. The incubation with primary antibody diluted in blocking buffer was performed in a cold room (4°C) overnight. Followed by incubation with Alexa Fluor®597/468 conjugated secondary antibodies, sections were then incubated with DAPI (1:1000, Sigma) for 2 mins. The primary antibodies used are as follows: mouse anti-SMαA (1:500, Sigma); goat anti-fluorescein (1:500, Abcam). Mouse or goat IgGs were used at the same concentration as control (**data not shown**). Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 20×, NA 0.5, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, and were processed with Photoshop software (Adobe).

Statistical analysis. Data were expressed as mean±SEM and analyzed using a two-tailed student's *t*-test for two-groups comparision or one-way ANOVA for comparing different groups. Chi-Square analysis was performed using SPSS 17.0 software to compare the percentage of embryonic maldevelopment in two groups. A value of $P < 0.05$ was considered as statistically significant.

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Supplementary Figures:

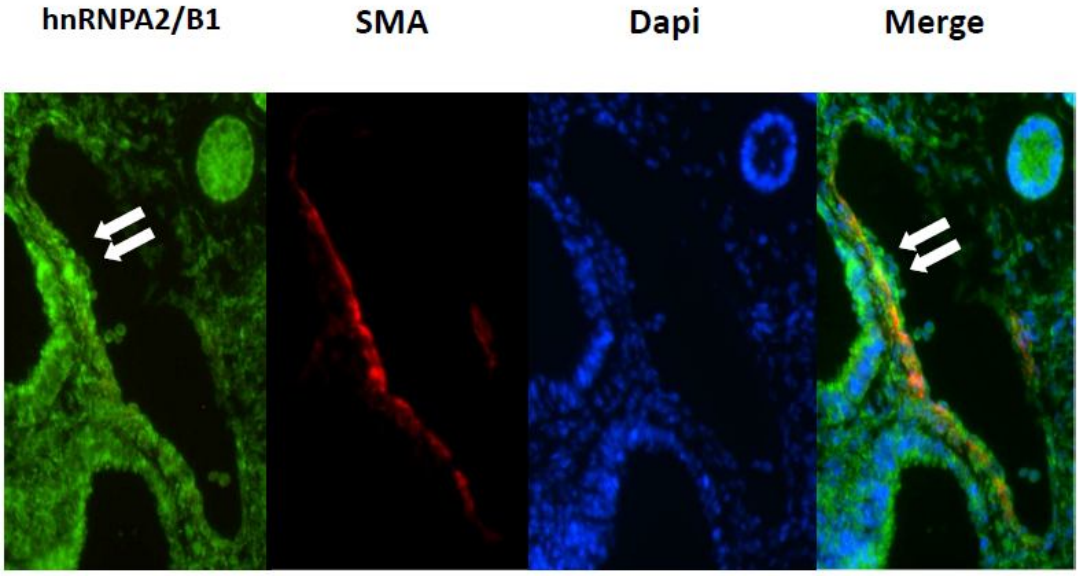

Figure S1. hnRNPA2/B1 was expressed on the floor of dorsal aorta. Double immunofluorescent staining on cryosection of HH16 chick embryo was performed with antibodies against hnRNPA2/B1 (green) and SMαA (red). Some green fluorescence labelled cells (white arrow) could be found in the floor of dorsal aorta and also expressed red fluorescence. Nucleus was counterstained with DAPI. Isotype IgG substituted primary antibody as negative control during staining process (data now shown).

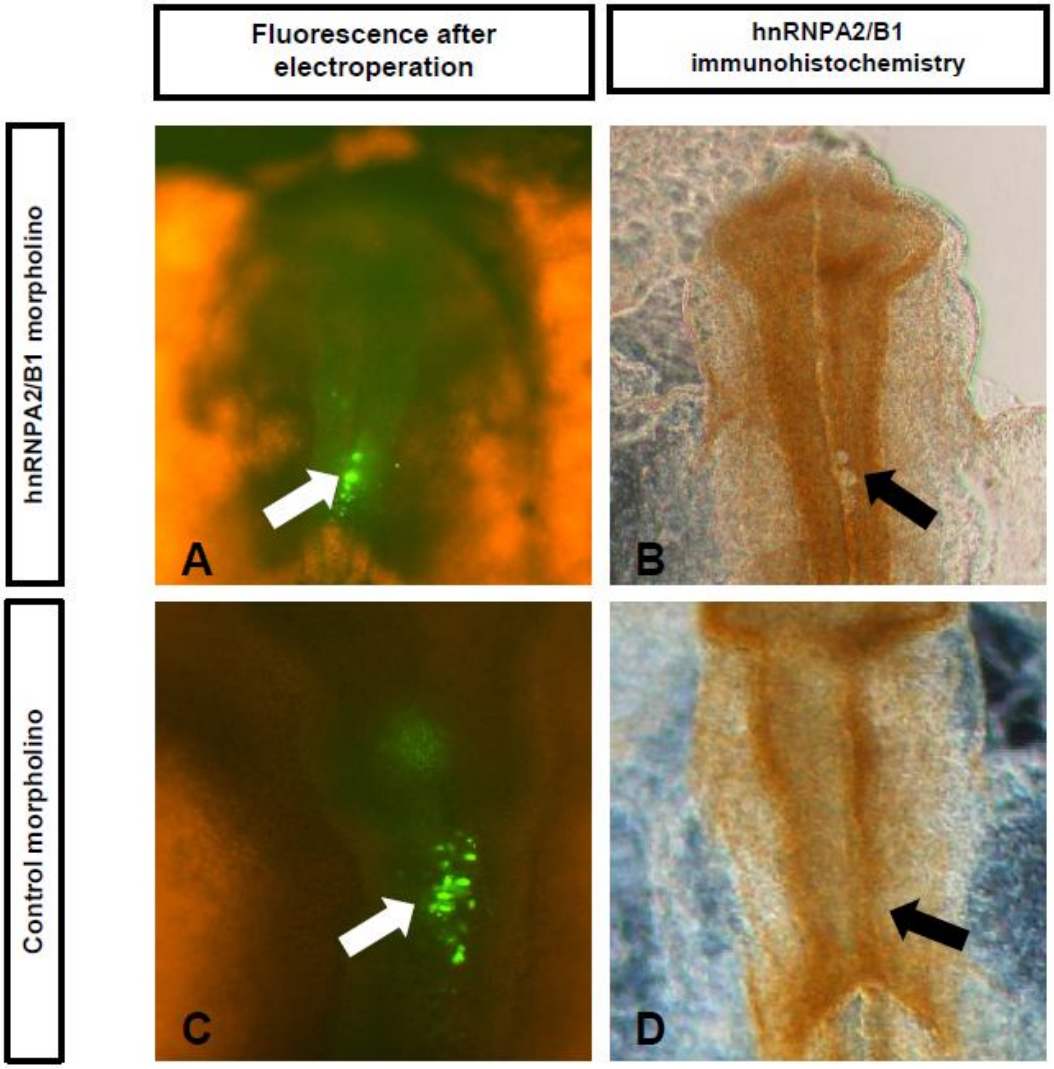

Figure S2. hnRNPA2/B1 morpholino oligonucleotide transfection inhibits its protein expression in chick neural tube. Specific designed hnRNPA2/B1 morpholino mixed with 60% sucrose was injected into HH9-10 chick embryonic neural tube by a pulled microcapillary. Electroporation was carried out as described in the Methods section. The labelling was observed under fluorescent dissecting microscope 3 hours after electroperation. White arrows indicate neural tube transfected with morpholino (A and C). Then the embryos were subjected to whole mount immunohistochemical staining for hnRNPA2/B1. Black arrow in (B) indicates a significant reduced intensity of hnRNPA2/B1 staining on the righ-hand side neural tube compared with to the left-hand side, while there is no difference in hnRNPA2/B1 staining intensity in control morpholino group (D). Isotype mouse IgG substituted primary antibody as negative control during the staining process (data not shown).

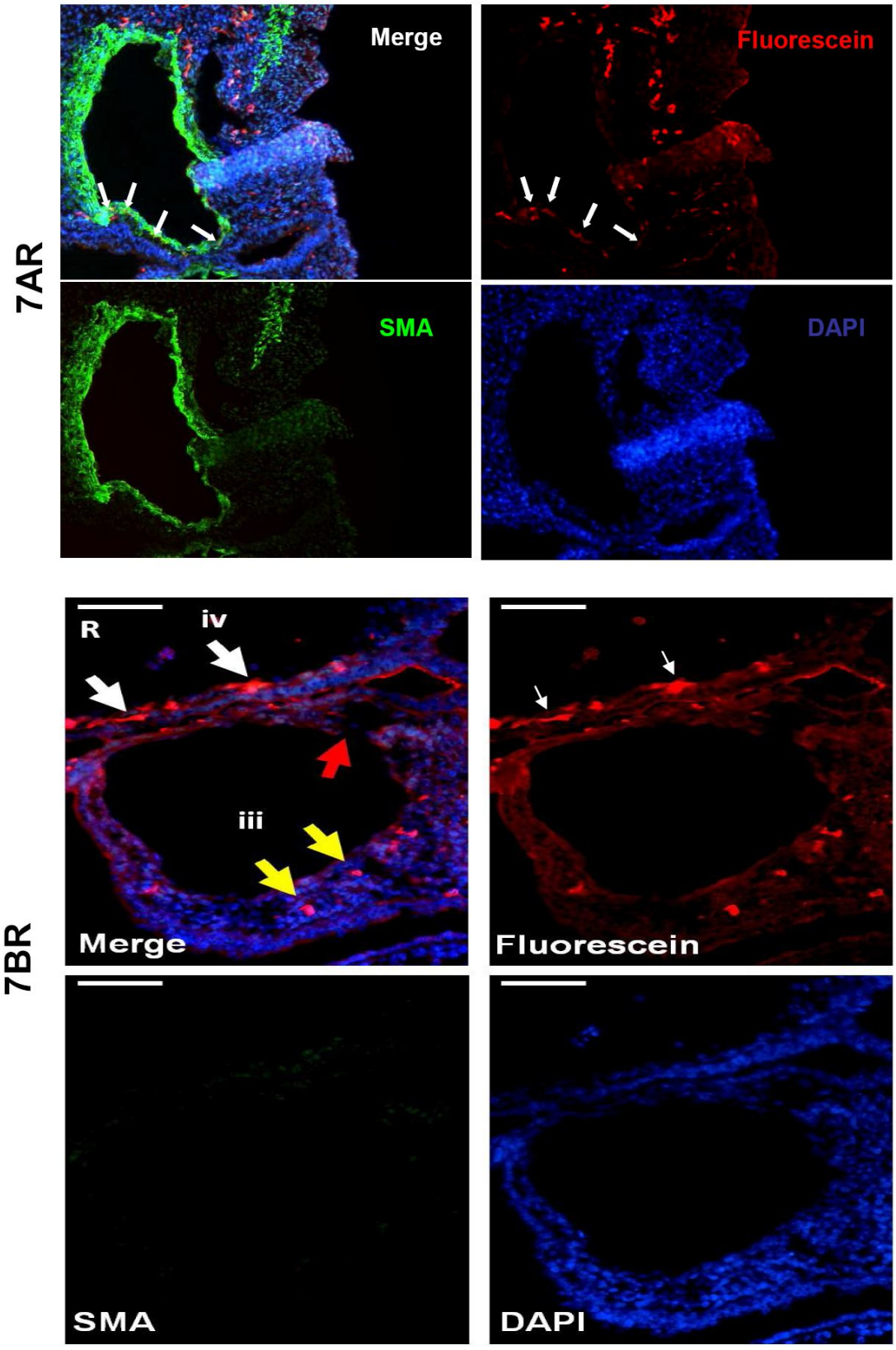

Figure S3. Un-merged images of Figure 7AR and 7BR

Supplementary table 1: Primer sets used in the present study

Gene names	Forward (5'-3')	Reverse (5'-3')	Application
18s	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	Real-time RT-PCR
SMαA	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG	Real-time RT-PCR
SM22α	GAT ATG GCA GCA GTG CAG AG	AGT TGG CTG TCT GTG AAG TC	Real-time RT-PCR
h1-Calponin	GGT CCT GCC TAC GGC TTG TC	TCG CAA AGA ATG ATC CCG TC	Real-time RT-PCR
SM-MHC	AAG CAG CCA GCA TCA AGG AG	AGC TCT GCC ATG TCC TCC AC	Real-time RT-PCR
SRF	CCTACCAGGTGTCGGAATCTGA	TCTGGATTGTGGAGGTGGTACC	Real-time RT-PCR
Myocardin	TCAATGAGAAGATCGCTCTCCG	GTCATCCTCAAAGGCGAATGC	Real-time RT-PCR
MEF2C	AAGCCAAATCTCCTCCCCCTAT	TGATTCACTGATGGCATCGTGT	Real-time RT-PCR
Cbx3	GAACGAATAATCGGCGCCA	ATGTTCGCCTCCTTTGCCA	Real-time RT-PCR
SMαA-P	GGCAACACAGGCTGGTTAAT	AGCCCCTGTCAGGCTAGTCT	CHIP assay
SM22α -P	GCAGGTTCCTTTGTCGGGCCA	CTGCTTGGCTCACCACCCCG	CHIP assay
pCMV5-HA-hnRNPA2/B1	GCTGAGGGTACCTTGGAGAGAGAAAAGGAACAG	GGAAGACTGCAGATATCTGCTCCTTCCACCATAG	hnRNPA2/B1 clone
pSPT18-hnRNPA2/B1 (Gallus)	GAACGGCTGCAGATGCCTCGCGGCGACAGAGAG	GCTTGCGGTACCTCAGTAACGGCTTCTCCCTC	Chick hnRNPA2/B1 clone